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## THE ACTION OF 1,2-EPOXIDES ON PROTEINS

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During a study of methods for the modification of waste proteins for industrial use, the scarcity of reagents known to combine with protein carboxyl groups became evident. Indications that the carboxyl groups of silk fibroin and of wool might be esterified by treatment with diazomethane (1) or methyl sulfate or halide (2) have been reported. The conditions of the usual methods for esterification, however, may be harmful for many proteins (3). It appeared that 1,2-epoxides ( $R-CH-CH-R'$ ) might

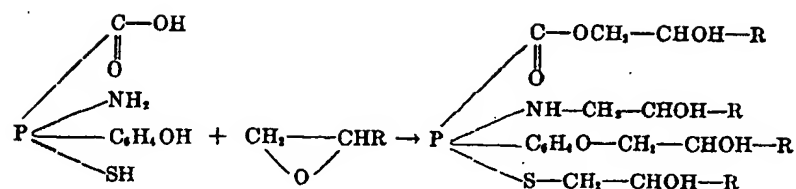


prove practicable, inasmuch as they are known to combine with acids under mild conditions (4). Several representatives of this class of compounds, such as ethylene oxide, 1,2-propylene oxide, and epichlorohydrin, are now commercially available. A search of the literature revealed no information concerning the nature of the interaction of these reagents with proteins, other than a patented procedure for the esterification of casein (5). The action of epoxides on amino acids appears to have been studied exclusively in anhydrous media (6, 7). Model experiments on the esterification of fatty acids and amino acids by epoxides in aqueous solution at room temperature were therefore initiated simultaneously with an investigation of the effect of these reagents on several proteins. Some results of the former experiments are being reported elsewhere.<sup>1</sup> They indicated that epoxides were effective esterifying agents for dissociated carboxyl groups. Amino groups, on the other hand, were found to combine with the reagent more rapidly when uncharged.

Treatment with epoxides will be shown here to be similarly effective for modifying proteins in aqueous solution at room temperature. While this publication will be confined to the studies performed on crystalline egg albumin and  $\beta$ -lactoglobulin, other proteins were found to react generally in a similar manner.<sup>1</sup> The reactions which were found to occur can be illustrated by the accompanying scheme.

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<sup>1</sup> Fraenkel-Conrat, H., Olcott, H. S., and Cooper, M., in preparation for publication.



The most pronounced effect on the properties of the protein is due to the esterification of a great proportion of its carboxyl groups. This causes a shift in the isoelectric point by 1 to 3 pH units. The solubility of the protein in neutral and alkaline solution is diminished and the electrophoretic mobility is affected.

The addition of the reagent to the amino groups does not suppress their basic nature and therefore does not tend to balance the effects of esterification of carboxyl groups on the properties of the protein.

Phenolic and sulfhydryl groups form ethers and thio ethers with the epoxide reagents. A study of the reaction of aliphatic hydroxyl groups is precluded by the lack of a test for these groups in proteins, but it may be assumed that aliphatic ethers do not form under the experimental conditions used, since the introduction of many new hydroxyl groups by combination of the reagent with all types of protein groups would then be expected to lead to unlimited polymerization. Actually the yield of the protein derivative is limited, the amount of reagent introduced never exceeding 10 per cent of the weight of the protein, regardless of the excess used.

#### EXPERIMENTAL<sup>1</sup>

*Preparation of Derivatives*—The reaction between epoxides and proteins was performed under various conditions, four of which offered definite and specific advantages. These were (1) reaction in neutral salt-free solution, (2) reaction in solution acidified with acetic acid to pH 3.5 (approximately 0.4 gm. of acid per gm. of protein),<sup>2</sup> (3) reaction in sodium hydroxide solution of pH 8 (approximately 2.7 ml. of 0.1 N alkali per gm. of protein), and (4) reaction in 6.6 M urea. The egg albumin samples used in the experiments were placed at our disposal by F. E. Lindquist and R. A. O'Connell of this Laboratory; they were prepared according to Kekwick and Cannan (8) and were recrystallized from two to four times. The crystalline  $\beta$ -lactoglobulin was kindly supplied by E. F. Jansen of this Laboratory who prepared it according to the method of Palmer (9). Ethylene and propylene oxides were commercial preparations; epichlorohydrin was furnished by the Shell Development Company.

<sup>1</sup> The details of representative experiments are summarized in Table I.

<sup>2</sup> Hydrochloric acid was found unsuitable since it combined more rapidly with the reagent than did the protein.

TABLE I  
Effect of Epoxides on Proteins; Experimental Details

Method No.	Conditions of treatment				Reaction product						
	Concentrations of			Time	Solubility	Yield	Total N	Amino N†	Fraction of original†		
	Protein	Ep-oxide*	Other reagents						Phenol (+ Indole)	Acid groups	Basic groups
	per cent	per cent	per cent	days		per cent	per cent	per cent	per cent	per cent	per cent
1	Egg albumin										
	3.3	13		1	Ppt.	100	14.2	0.22	63	54	
	1.46	17		4	"	107	13.4	0.15	36	26	109
	1.64	6.4		2	"	93	14.5	0.21	<43†	49	
2	1.67	4.8*		2	"	94	14.3	0.17	<56†	38	
	2.05	14	Acetic acid, 0.75 (pH 3.5)	2	Soluble	90	14.7	0.60	<100†	65	137
	1.52	13	Acetic acid, 0.61	6	"	92	14.6	0.44	<56†	52	
	3.0	12	NaOH, 0.04 (pH 8.1)	1	Gel	101	13.9	0.03	64	55	
3	0.93	13	NaOH, 0.01	2	Soluble	107	13.5	0.05	27	26	106
	2.2	9.5	" 0.03	7	"	112	13.6	0.03	27	18	112
	1.67	14.3	NaCl, 0.08	2	Ppt.-gel	116	13.7	0.05	50	31	
	2.1	8.3	Urea, 40	1	Soluble	96	13.9			60	
4	0.93	13	" 40	2	"	107	13.7	0.06	<40†	30	100
	1.19	13	" 40	4	"	120	13.8	0.03	<28†	20	
	1.19	13	" 40	7	"	110	13.7	0.03	20	22	
	$\beta$ -Lactoglobulin										
3	6.4	20	NaCl, 0.8	1	Ppt.-gel	12.9	0.12		76	44	
	3.1	14	" 0.4	2	Soluble	91	12.9			37	
	6.7	17	" 0.8	4	Ppt.-gel	88	12.7	0.18	48	29	111
	Untreated egg albumin					15.0	0.63		100	100	100
	Untreated $\beta$ -lactoglobulin					15.0	1.25		100	100	100

\* Propylene oxide was used in all experiments except that marked with an asterisk in which ethylene oxide was used at a concentration equivalent to that of propylene oxide in the preceding experiment.

† None of these data is corrected for the increase in mass of the protein through combination with the reagent.

‡ These results were obtained from readings of turbid solutions.

More work was done with propylene oxide than with the lower boiling ethylene oxide or the water-insoluble epichlorohydrin. A comparison of the properties of egg albumin derivatives prepared by the use of equimolar

amounts of the three reagents indicated no major difference, with the exception that the epichlorohydrin-treated material was less soluble than the other two.

When an epoxide was added to a dialyzed egg albumin solution (1.5 to 4 per cent), a turbidity appeared within a few seconds, followed by gradual precipitation of the protein (Method 1). Complete precipitation of the derivative occurred when the reagent concentration was 10 to 20 per cent; only 30 to 50 per cent of the protein was precipitated at epoxide concentrations of 3 and 5 per cent. The insoluble protein derivative could be isolated by centrifugation. Repeated washing with 0.1 M sodium chloride solution did not cause losses of the material exceeding 5 per cent.

When the treatment was carried out in acid or alkaline solution, or in urea (Methods 2, 3, 4), no precipitate formed even upon prolonged standing. The alkaline solutions were slowly transformed into clear gels if the protein concentration was high (3 to 4 per cent). When the protein was treated with propylene oxide in 0.5 to 1.0 per cent sodium chloride solution, a precipitate formed and again disappeared within about 16 hours, resulting in a clear solution or gel, depending upon protein concentration. This phenomenon could be explained as due to the known tendency of epoxides to add hydrochloric acid, even from neutral solutions (4), with the formation of free hydroxyl ions. Propylene oxide treatment in salt solution therefore corresponded to that performed with added alkali (Method 3), as was borne out by the physical and chemical properties of the resulting protein derivatives.

Regardless of the nature of the solvent, the reaction of proteins with epoxides was found to be accompanied by a shift in the pH of the reaction mixture toward the alkaline side. This shift had previously been observed also with amino acids<sup>1</sup> and is a direct consequence of the esterification of carboxyl groups.

To isolate the reaction products, the solutions (or gels) were dialyzed; this increased the tendency toward gel formation. Electrodialysis was then found convenient, leading to precipitation of the entire modified protein, regardless of its previous physical state. The salt-free suspensions could be centrifuged satisfactorily, notwithstanding the gelatinous appearance of the electrodialyzed protein. The precipitates were frozen and dried *in vacuo*, yielding the modified protein in the form of a light powder, generally in yields of 100 to 110 per cent. While the preparation of the protein derivatives by these methods was slightly more laborious than by precipitation from neutral solution (Method 1), it offered definite advantages. Thus treatment of the protein in alkali or in urea favored the reaction of the amino groups with the reagent. Variations in the conditions of treatment also affected the solubility of the protein derivative, as will be demonstrated in the following section.

*Solubility of Derivatives*—As indicated by the methods of isolation, all protein derivatives were almost completely insoluble in distilled water and in dilute salt solutions. They were, however, soluble in 40 per cent urea. Their solubility in acid and alkaline solutions corresponded to what might be expected of protein derivatives containing fewer acid groups but the original number of basic groups. Thus all derivatives studied were soluble in dilute acid, but much less soluble or insoluble in alkali.

Preparations obtained by Method 1 were the least soluble. They could be dissolved only at approximately pH 3, or at pH 2.5 if epichlorohydrin had been used in their preparation. Subsequent addition of alkali gave rise to a turbidity followed by precipitation when the pH rose to 4.5 to 6.5, depending upon protein concentration. Addition of alkali up to pH 11 or 12 generally resulted in resolution of the material, but the product could not be dissolved by the same amount of alkali directly; i.e., without previously having been dissolved in acid. An explanation for this was suggested by the finding that a small part of the ester linkages was not stable in acid solutions, as will be shown below.

The derivatives prepared in acid or alkali (Methods 2 and 3) were more soluble than those obtained by Method 1. They could be dissolved directly in alkali as well as in a weakly acidic medium (at pH 11 or 6, to 1 per cent or more). The same was the case for preparations obtained by Method 4 if treatment was restricted to a period of 1 or 2 days only. After 4 days treatment in urea, the product was considerably less soluble.

*Isoelectric Point of Derivatives*—The fact, already mentioned, that epoxide-treated egg albumin and  $\beta$ -lactoglobulin preparations were least soluble between pH 7 and 8 suggested a shift of their isoelectric points by about 2 to 3 pH units. Owing to their insolubility in the isoelectric region, indirect methods were used in search of confirming evidence. Electro-dialyzed preparations of both treated (Method 4, 4 days) and untreated egg albumin were dissolved in 6.6 M urea (to 1.5 per cent) and the pH values of these solutions were determined by means of a glass electrode. These were pH 5.7 for the untreated and pH 8.7 for the treated sample. While both these values were slightly higher than expected, possibly owing to an error in the pH measurements introduced by the solvent (40 per cent urea), the difference may be regarded as further evidence for a shift of the isoelectric point of the treated protein.

Confirmation was also obtained from studies of the dye-combining capacity of the proteins and their derivatives. Chapman, Greenberg, and Schmidt (10, 11) showed that negatively charged protein groups combined with basic dyes and positively charged groups with acid dyes. On the basis of the assumption that the isoelectric point of a protein corresponds to the pH at which the capacity of the protein to combine with both acid and basic dyes is at a minimum, the use of dyes has been suggested for the

approximate determination of the isoelectric point of soluble and insoluble proteins.<sup>1</sup> It was observed that the esterified derivatives of both egg albumin and  $\beta$ -lactoglobulin bound no measurable amounts of the acid or basic dyes between pH 6 and 8, while the untreated proteins bound no dye at pH 5.

Further confirmation of the change in the isoelectric point of the treated proteins came from electrophoretic experiments.

*Electrophoretic Migration*—These experiments were kindly performed by Dr. H. P. Lundgren and Dr. W. Ward of this Laboratory by the conventional Tiselius technique. The results of representative runs, summarized in Table II, show that propylene oxide-treated egg albumin and  $\beta$ -lactoglobulin migrated toward the cathode at pH 5.8 and 6.0, while the un-

TABLE II  
*Effect of Propylene Oxide Treatment on Electrophoretic Mobility of Proteins*

Protein and type of treatment	Electrophoretic mobility*	pH†
	sq. cm. per volt sec. $\times 10^6$	
Egg albumin, untreated.....	-4.4	5.8
" " Method 1, 1 day.....	+3.0	5.5
" " " 2, 7 days.....	+5.1	5.8
" " untreated.....	-8.8	9.5
" " Method 4, 1 day.....	-6.2	9.6
" " " 3, 7 days.....	-4.1	9.5
$\beta$ -Lactoglobulin, untreated.....	-2.9	5.9
" Method 3, 1 day.....	+0.73	6.0

\* Descending boundary.

† Acetate buffers were used at pH 5.5 to 6.0; a glycine buffer at pH 9.5 to 9.6. The ionic strength was 0.03 to 0.04 throughout.

treated proteins migrated anodically at this reaction. At pH 9.6 to 9.9 all samples migrated toward the anode, the treated ones, as might be expected, at a lower rate than the untreated preparations. With regard to homogeneity, the epoxide derivatives included in Table II appeared to be of the same order of "purity" as the untreated proteins. The latter may have contained as much as 20 per cent of a contaminant of similar mobility as the main fraction, as indicated by a single but not quite symmetrical boundary. In contrast to these more soluble epoxide derivatives obtained by Methods 2, 3, and 4, most of those prepared according to Method 1 had to be run in the Tiselius apparatus below pH 4, owing to their insolubility in salt solutions of higher pH. These latter preparations showed inhomogeneity to a considerable extent, due perhaps to the method

of preparation or to an action of the buffers of pH 2.5 and 3.5 on the protein derivatives.

#### Determination of Protein Groups

*Amino, Phenolic, and Sulfhydryl Groups*—Amino groups were estimated by the Van Slyke manometric procedure (12), with a 15 minute reaction period in a chamber protected from light (13). The amino nitrogen content of various protein preparations was found to be largely independent of the physical state of the material; thus, similar values were obtained

TABLE III  
Effect of Propylene Oxide Treatment on Protein Groups

	Method No.	Conditions of treatment		Decrease in groups		
		Medium	Days	Carboxyl	Amino	Phenol (+ indole)
Egg albumin + propylene oxide	1	Neutral	1	44	63	34
			2	49	66	55
			4	70	73	60
	2	Acid	2	34	2	>0
			6	46	27	>42
			1	41	95	31
	3	Alkali	2	68	89	66
			7	78	95	70
			2	59	91	50
	4	Urea	2	67	87	>55
			4	78	95	>69
			7	76	95	78
Egg albumin + ethylene oxide	1	Neutral	2	60	71	41
	4	Urea	2	77	85	80
$\beta$ -Lactoglobulin + propylene oxide	3	Saline	1	49	88	12
			4	66	72	43

\* Corrected for the increase in mass of the protein through combination with the reagent, as indicated by decreases in nitrogen content (see Table I).

for suspensions of 60 to 200 mesh powders and for acid solutions of the same material. Amino nitrogen determinations of various epoxide-treated preparations indicated that the amino groups of proteins had reacted almost quantitatively when the treatment was performed in alkali or in urea (Methods 3 and 4). The derivatives prepared by rapid precipitation from neutral solution (Method 1) still retained about 30 to 40 per cent, and those prepared in acid (Method 2) 70 to 100 per cent, of their original amino groups (Table III). These findings are in agreement with those of model experiments on amino acids,<sup>1</sup> which indicated that the amino groups reacted preferentially in alkaline solution.

Other protein groups for which test methods were available were the phenol (plus indole) groups and the sulfhydryl groups. For these colorimetric tests, the protein had to be used in solution rather than in suspension. The Herriott modification (14) of the Folin method was employed for the estimation of the phenolic groups. The readings obtained with protein derivatives were compared with a standardization curve prepared with 2 to 20 mg. of native egg albumin.<sup>4</sup> Upon addition of the reagent and buffer, some of the samples studied yielded turbid solutions which could not be clarified by centrifugation. No attempts were made to avoid this by the use of a solvent, such as urea or a detergent, which would have introduced the unknown factor of the effect of denaturation through the solvent on the available tyrosine groups (15). Clear solutions were obtained with a sufficient number of samples to permit the conclusion that the phenolic groups had been blocked to about 80 per cent by both propylene oxide and ethylene oxide in urea solution, slightly less in alkaline and neutral solution without urea, and least in acid solution (Table III).

Sulfhydryl tests with the Folin reagent in urea solution (16) were negative for the derivatives of both egg albumin and  $\beta$ -lactoglobulin. Thus it appeared that the original protein thiol groups had been transformed into thio ethers.

*Acid and Total Basic Groups*—While the apparent changes in solubility, isoelectric point, and electrophoretic behavior of the treated proteins strongly suggested that the carboxyl groups had been esterified, further proof and in particular a quantitative measure of this reaction had as yet to be found. Titration curves were determined repeatedly, but, owing to the extended range of insolubility of the modified proteins, these curves were necessarily of only limited significance.

The finding of Chapman, Greenberg, and Schmidt (10, 11) that acid and basic dyes combined stoichiometrically with basic and acid protein groups supplied a convenient tool for the estimation of these groups. An application of this finding to a procedure suitable for routine analysis is described in the following paper. With this method the number of acid groups of proteins was found markedly decreased by treatment with epoxides, indicating the esterification of 45 to 78 per cent of the original carboxyl groups. Treatment for several days in alkaline or urea solution (Methods 3 and 4) led to the disappearance of a slightly larger proportion of the carboxyl groups than treatment in neutral solution (Method 1); the acid medium (Method 2) was least favorable for esterification. Ethylene oxide was about as effective as propylene oxide in decreasing protein carboxyl groups.

<sup>4</sup> This curve was not a straight line, in contrast to that for free tyrosine from 0.15 to 0.5 mg.

Determinations by dye methods of the total basic groups of various propylene oxide-treated protein preparations yielded results which were similar to or higher than those obtained with the untreated proteins (Table I).<sup>6</sup> Thus conversion of the primary amino groups to secondary and possibly tertiary isopropanol amines did not affect their basic nature sufficiently to prevent them from combining with an acid dye at pH 2.2.

*Elementary Analyses of Derivatives*—In view of the finding that epoxides reacted with a great number of protein groups, it was thought that the consequent depression in the nitrogen content of the protein derivatives might represent a quantitative measure of the over-all extent of interaction. For this purpose electrodyalyzed samples were analyzed by the Kjeldahl method. The nitrogen content of untreated egg albumin was found to be 15.0 per cent. That of treated preparations ranged down to 13.4 per cent (Table I).<sup>6</sup> These data indicated that the amount of epoxides combined with egg albumin represented as much as 9 per cent of the weight of the derivative. From this it can be calculated that 80 moles of propylene oxide may be bound by 1 mole of egg albumin, which corresponds to the number of protein groups found blocked by analyses, namely four-fifths of the carboxyl and phenolic groups (forty-one and eight respectively) and all of the amino and sulfhydryl groups (twenty-two and seven respectively).<sup>7</sup> It must be recognized, however, that these calculations are necessarily only approximations, since 1 or 2 moles of the reagent may combine with the primary amino groups of proteins. The former was assumed in the present calculations.

The nitrogen content of  $\beta$ -lactoglobulin<sup>8</sup> was depressed from 15.0 to 12.7 per cent through propylene oxide treatment. This corresponded to the introduction of 120 moles of propylene oxide into each mole of protein. However, the number of moles accounted for by protein group analyses was only 84.<sup>7</sup>

<sup>6</sup> The apparent increases in the basic groups of proteins upon esterification are not yet understood.

<sup>7</sup> Determined after drying at 105° for 16 hours. When the moisture was determined separately and correction applied, the nitrogen content of the untreated egg albumin was 15.75 per cent. The apparent discrepancy appears to be due to the hygroscopicity of the material as recently emphasized by Chibnall, Rees, and Williams (17). Although the samples analyzed by the routine method were probably not dry, they have been regarded as containing similar moisture contents, since epoxide treatment does not affect the hydrophilic nature of proteins (see foot-note 1).

<sup>8</sup> These calculations are based on recent estimates of the molecular weights and of the number of carboxyl groups per mole for egg albumin and  $\beta$ -lactoglobulin, as summarized by Cohn and Edsall (3) (mol. wt. 45,000, with 51 carboxyl (including phosphoric acid) groups for egg albumin; mol. wt. 40,000, with 58 carboxyl groups for  $\beta$ -lactoglobulin). Data for the amino groups, tyrosine and cysteine, of egg albumin were taken from the same source. The corresponding values for  $\beta$ -lactoglobulin are those of Brand and Kassell (18).

The results of analyses for the chlorine content of epichlorohydrin-treated proteins which had not been electrodialed could be similarly interpreted. Electrodialyzed samples, however, contained considerably less chlorine than would be expected from their nitrogen content. It appeared probable that this procedure may have caused a loss through hydrolysis of part of the chlorine. The chlorine was also found to be removed readily by alkali.

*Stability of Protein-Epoxy Bonds toward Acid and Alkali*—It appeared of importance to ascertain the lability of the newly formed bonds toward acid or alkali, particularly in view of the fact that most protein group analyses were performed on samples that had been dissolved by means of acid or alkali or both. For this purpose various samples of propylene oxide-treated egg albumin were dissolved in 0.01 or 0.025 *N* hydrochloric acid or sodium hydroxide,<sup>8</sup> at protein concentrations of 0.75 and 1.875 per cent respectively. The solutions had pH values of 2.4 and 11.5. The numbers of free tyrosine, thiol, amino, and carboxyl groups were determined immediately and again after 1 or 2 days incubation at 40°.

Comparison of these values indicated in general little change.<sup>9</sup> A slight hydrolysis of esterified carboxylic esters seemed to occur in about half of the experiments, but the changes were of the same order as the error of the method used (10 per cent). An increase of the free phenolic groups was observed only in one out of seven experiments. The primary amino groups were found to be partly regenerated in alkali, but never in acid. This alkali lability of some isopropanol-amino linkages also became evident upon comparing the amino nitrogen content of acid-dissolved with that of the directly alkali-dissolved protein derivatives; the latter value was consistently higher than the former (about 15 *versus* 5 per cent of the amino nitrogen of untreated egg albumin). A similar, though inverse, relationship seemed to hold for a small fraction of the carboxyl groups; these appeared to be released immediately upon solution in acid (at pH 2.5 to 3.0). This could be demonstrated with protein samples that had been treated with propylene oxide in alkaline solution (Method 3) and could thus be dissolved directly in alkali. It was also indicated by comparing the free acid groups of a preparation obtained by Method 4, when dissolved in the minimum amount of acid (1.67 ml. of 0.1 *N* HCl per gm. of protein, pH 5.5) and when dissolved in the usual amount (13.3 ml. of HCl, pH 2.4). In these experiments about 10 per cent more free carboxyl groups was found in the samples exposed to pH 2.4 than in those dissolved at pH 5.5 or 11.

<sup>8</sup> Samples not directly soluble in alkali were first dissolved in acid, and then treated with the necessary amount of alkali, added rapidly.

<sup>9</sup> The thio ether bonds of epoxide-treated proteins thus differ in their stability from the previously described thioacyl bonds (19).

## SUMMARY

Epoxides, such as ethylene oxide, propylene oxide, and epichlorohydrin, were found to be suitable reagents for the esterification of protein carboxyl groups in aqueous solution at room temperature.

Through treatment of crystalline egg albumin and  $\beta$ -lactoglobulin with these compounds, preparations of modified protein were obtained which differed from the original material in that:

1. The derived proteins showed isoelectric points which had been shifted as much as 3 pH units toward the alkaline side.
2. They were insoluble in the isoelectric region, both in distilled water and in salt solutions.
3. They were more soluble on the acid side than on the alkaline side of the isoelectric point.
4. They contained considerably fewer free carboxyl, phenolic, primary amino, and sulfhydryl groups than the untreated proteins. The decreases of these various types of groups varied greatly, but in a predictable manner, depending upon the conditions of treatment.
5. Their nitrogen contents depended upon the type and length of treatment. The lowest values observed with the two proteins were 13.4 and 12.7 per cent of nitrogen, which indicated the introduction of approximately 80 and 120 moles of reagent per mole of egg albumin and  $\beta$ -lactoglobulin, respectively. In the case of egg albumin, the calculated figure agreed with that to be anticipated from the number of substituted groups as found by the various analyses. With  $\beta$ -lactoglobulin the number of reagent residues accounted for by group analyses was lower than that indicated by nitrogen analysis.

The only property of the proteins which was not appreciably affected by the treatment was the number of their total basic groups. Thus any difference in the basic character of the original amino groups and of the newly formed imines did not reveal itself under the conditions of the test.

In general, the newly introduced bonds were surprisingly stable in acid and alkaline solution with the exception of small proportions of the substituted carboxyl and amino groups which were hydrolyzed readily in acid and alkali, respectively.

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*Addendum*—Treatment of a solution of egg albumin with propylene oxide at 95° (in a sealed tube) for 8 hours yielded one-half of the protein as a water-soluble derivative containing twice as many isopropanol residues as could maximally be introduced at room temperature. A considerable decrease in the total number of basic groups was observed, besides the usual loss of carboxyl and phenolic

groups, which suggested the possibility that these groups might have become completely substituted under such conditions. The effects of epoxide treatment at elevated temperature will be further investigated.

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